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Maintenance of heterocyst patterning in a filamentous cyanobacterium

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In the absence of sufficient combined nitrogen, some filamentous cyanobacteria differentiate nitrogen-fixing heterocysts at approximately every 10th cell position. As cells between heterocysts grow and divide, this initial pattern is maintained by the differentiation of a single cell approximately midway between existing heterocysts. This paper introduces a mathematical model for the maintenance of the periodic pattern of heterocysts differentiated by *Anabaena* sp. strain PCC 7120 based on the current experimental knowledge of the system. The model equations describe a non-diffusing activator (HetR) and two inhibitors (PatS and HetN) that undergo diffusion in a growing one-dimensional domain. The inhibitors in this model have distinct diffusion rates and temporal expression patterns. These unique aspects of the model reflect recent experimental findings regarding the molecular interactions that regulate patterning in *Anabaena*. Output from the model is in good agreement with both the temporal and spatial characteristics of the pattern maintenance process observed experimentally.

Keywords: pattern formation; reaction diffusion; bacteria; *Anabaena*

AMS Subject Classifications: 92C15; 92C80; 92-04; 35Q80

1. Introduction

A fundamental paradigm of developmental biology is the establishment of a regular pattern of differentiated cells from a group of equivalent cells. A subgroup of developmental patterns specifies within a field of cells the differentiation of cells into structures equidistant from one another. The generation of such periodic patterns has been described mathematically by reaction–diffusion equations, and the structures formed include sensory bristles in *Drosophila* [22], hair-like trichomes on the surface of *Arabidopsis* leaves [12], and heterocysts in filaments of the cyanobacterium *Anabaena*. In the latter, individual cells differentiate at approximately 10-cell intervals into nitrogen-fixing heterocysts that supply the remainder of cells with fixed nitrogen when little is available in the surrounding medium (Figure 1(a)). The one-dimensional pattern of only two

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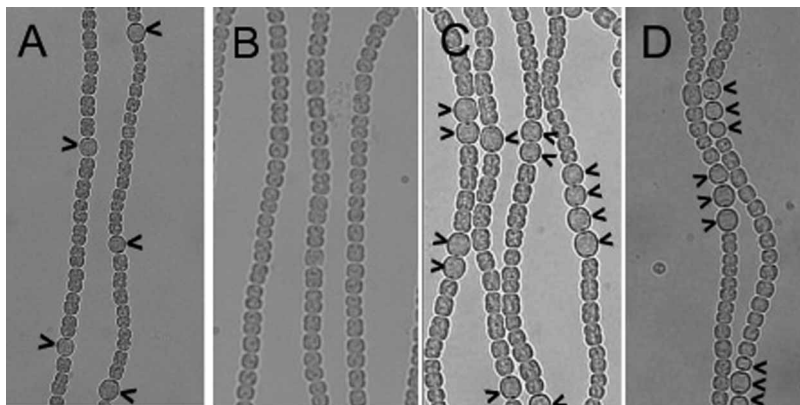


Figure 1. Patterns of heterocysts in *Anabaena* with different genetic backgrounds. (a) Wild-type *Anabaena* 24 h after the induction of differentiation by removal of combined nitrogen forms a periodic pattern of heterocysts. (b) Wild-type *Anabaena* in the presence of combined nitrogen, nitrate in this case, lacks heterocysts. (c) A mutant strain with *patS* deleted from the chromosome forms extra heterocysts 24 h after the induction of differentiation. (d) A mutant strain with *hetN* deleted from the chromosome forms extra heterocysts 48 h after the induction of differentiation. Carets indicate heterocysts.

cell types and the genetically tractable nature of the organism make it an attractive system for investigation of periodic patterns in biology.

Heterocysts are terminally differentiated cells that do not divide. About 12 h after the induction of heterocyst formation by transfer to a medium lacking fixed nitrogen, cells irreversibly commit to differentiating into heterocysts, and about 18 h after induction heterocysts are morphologically distinguishable from surrounding undifferentiated ‘vegetative’ cells and become functional at about 24 h [30]. Nitrate or ammonium in adequate concentrations suppresses the formation of heterocysts, in which case the filament composed exclusively of vegetative cells (Figure 1(b)). The elaboration of heterocysts facilitates the spatial separation of an oxygen-labile metabolic process, nitrogen fixation, from one that evolves molecular oxygen, photosynthesis with photosystem II (PS II). Fixed nitrogen is supplied to vegetative cells from heterocysts, and in return, heterocysts receive a source of carbon and reductant to compensate for their lack of PS II and the Calvin cycle.

Patterning of heterocysts along filaments can be divided into two discrete stages. Initial, *de novo* pattern formation takes place when filaments composed completely of vegetative cells are transferred to a medium lacking fixed nitrogen and a pattern forms in the absence of any previous pattern. Maintenance of this pattern occurs thereafter as the vegetative cells grow and divide, and a vegetative cell midway between two heterocysts differentiates to preserve, or maintain, the initial pattern. Together, *de novo* and maintenance patterning ensure an optimal ratio of heterocysts to vegetative cells over small spatial scale, which provides efficient exchange of metabolites between the two cell types.

Certain characteristics of the *Anabaena* system make it an ideal candidate for theoretical studies, including its one-dimensional characteristics and the potential for directed experimental and theoretical comparisons. Moreover, pattern formation in living organisms has been the subject of growing interests, with models proposed for the different levels of complexity. A mathematical model of this system needs to account for the diffusion of components from one cell to another associated with the non-homogenous spatial patterning. Reaction–diffusion equations known as the Turing equations or systems were originally developed by Turing [25] for the case of two concentration variables. Forms of these reaction diffusion equations have been a cornerstone of pattern formation studies in mathematical biology. In a series of studies spanning several decades, Gierer and Meinhardt [15] investigated the role of a short-range activator and a long-range inhibitor

in the pattern formation process. A pioneering, preliminary effort of modelling pattern formation in *Anabaena* followed directly from the use of reaction–diffusion equations in a study done by Wolk [27]. His early experimental studies suggested that heterocyst formation was mediated by the diffusion of genetic products and impaired by inhibition. He proposed a model for pattern initiation based on the reaction–diffusion equations that offered some qualitative agreement with the available data at that time, which was before any of the regulatory proteins had been identified. The roles of inhibition and diffusion were further highlighted in a subsequent study by Wilcox *et al.* [26], though explicit equations were not provided. Computations of reaction and diffusion equations associated with the system were presented using an L systems network model by Barker and Herman [2]. A related mathematical model for pattern maintenance was introduced by De Koster and Lindenmayer [11]. They assumed the patterns resulted from the diffusion of a single, unspecified inhibitor. They presented some analytical solutions for the diffusion along with complementary numerical simulations. Their efforts provide some useful broad insights; however, their work is not well known or cited by the biology researchers. This may be in part due to the fact that they did not specify the underlying inhibitor, which was probably due to limited knowledge of the patterning proteins at the time of their study. Unlike the work by Wolk, De Koster and Lindenmayer incorporated domain growth into their model in an approximate manner. Subsequent and more comprehensive work has investigated the role of domain growth on the reaction–diffusion equations and has reported mode-doubling pattern transitions similar to those observed in *Anabaena* may occur [3]. We also note that recent work has examined initial pattern formation with respect to the role of nitrogen as in the work by Allard *et al.* [1] who considered growth and division without patterning proteins. Their biological system is very different from the wild-type organism we study in this paper.

Refined and extended models have been introduced to account for the unique spatial pattern characteristics. Meinhardt [19] has suggested that pattern formation in *Anabaena* may follow from a novel, non-Turing mechanism in which only an inhibitor diffuses from cell to cell in the presence of a diffusive activator. Meinhardt has conceptually illustrated this mechanism in conjunction with the domain growth of the array of cells. Heterocyst formation follows when the inhibitor, PatS in this case, drops below a threshold level and autocatalysis of the activator is initiated. This formulation of the maintenance mechanism has been described with figures, but the corresponding equations and parameters have not been provided. The pattern formation mechanisms suggested by Meinhardt are intriguing and in part provide motivation for this investigation. Recently, a gene network model has been introduced using a fixed array of cells arranged cyclically [14]. The gene circuit consisted of interaction among *ntcA*, *hetR* and *patS*. The model predicts results similar to experimental observations in terms of the effect of initial nitrogen deprivation and spacing between heterocysts.

All of these mathematical studies, even with approximate and limited formulations, have given new insights; however, each supposes the existence of only one diffusible inhibitor. In this study, we introduce a model that includes two inhibitors, PatS and HetN, which diffuse at significantly different rates. This novel extension is motivated by experimental studies, which have demonstrated the presence of the second inhibitor [5,9]. The behaviour of the two inhibitors with an activator has been examined in the context of reacting chemical systems but only for cases in which all three components undergo diffusion [13].

The biological motivation for this formulation is fundamental to our study. Thus, it is important to highlight our selection of the interactions in the system. We first outline the role of HetR. Early models incorporated the diffusion of HetR; however, there is no evidence for this, and experimental results are consistent with a lack of diffusion of HetR. In the regulatory network controlling heterocyst differentiation and patterning, the protein HetR is an activator of differentiation that seems to have many of the characteristics of activators described in reaction–diffusion systems capable of producing periodic patterns. HetR is a transcriptional activator protein that positively

autoregulates its own transcription as well as being necessary for transcription of genes encoding two inhibitors of differentiation, PatS and HetN [16]. HetR acts as a dimer, thus providing nonlinearity to its activity. Without HetR, filaments are incapable of differentiating heterocysts, and with extra HetR, filaments differentiate even in the presence of ammonium, conditions under which heterocysts are not normally formed [8]. Thus, extra amounts of HetR can bypass the normal induction of differentiation by a lack of combined nitrogen.

The inhibitor that has been considered in previous studies is PatS. The *patS* gene is predicted to encode a 13 or 17 amino acid protein that is presumably processed to a smaller, active form, perhaps during export from the cytoplasm to the periplasm of the cell or directly to a neighbouring cell [29]. Two recent reports have demonstrated that molecules the size of PatS freely diffuse from cell to cell through a continuous periplasm, the region between the inner and outer membranes of Gram-negative bacteria, or through intercellular junctions [18,20]. Demonstration of a possible route for the diffusion of regulatory proteins provides for the formation of concentration gradients of regulatory proteins, a requirement for a reaction–diffusion-mediated process. The active form of PatS has yet to be identified, and a presumed gradient of PatS has not been demonstrated. However, when confined to the cytoplasm of the cell that produced it, PatS is incapable of restoring a normal pattern of heterocysts to a *patS*-mutant strain, suggesting that it must diffuse from cell to cell to function properly in cell patterning [28]. In addition, the expression of *patS* in only one or two cells of a filament was recently shown to lower the levels of HetR protein in neighbouring cells [23]. Fluorescence from a HetR–GFP fusion protein was undetectable in adjacent cells and increased with distance from cells overexpressing *patS*, providing the most concrete evidence that a PatS-dependent signal diffuses from cell to cell. A peptide corresponding to the predicted C-terminal 5 amino acids of PatS (PatS-5; RGSGR) prevents the DNA-binding activity of HetR *in vitro* [16], and its addition to the medium prevents differentiation of heterocysts [29]. The small size of a functional PatS peptide suggests a fast rate of diffusion. A mutant strain lacking PatS has reduced spacing between heterocysts, and adjacent cells often differentiate to give multiple contiguous heterocysts (Mch phenotype; Figure 1(c)). Two transcriptional start points (tsps) have been identified in the promoter region of *patS* [30]. One tsp is developmentally regulated to give increased transcription in developing cells, and the other appears to be active in vegetative cells and provides a basal level of transcription under all conditions of growth. After differentiation of an initial patterned group of heterocysts, the expression of *patS* has been shown to revert back to the baseline level seen in filaments before the induction of differentiation [29].

The second inhibitor, included here for the first time in a mathematical model, is HetN. HetN is not necessary for proper *de novo* pattern formation, but it is instead necessary for stabilization and maintenance of the pattern as the filament enlarges due to vegetative cell growth and division [9]. Unlike mutant filaments lacking PatS, which has a disturbed *de novo* pattern of heterocysts, a mutant lacking HetN has a delayed Mch phenotype; the pattern is normal at 24 h after induction, the approximate time from induction to formation of initial heterocysts, but is Mch after 48 h (Figure 1(d)), approximately twice the time from induction to the formation of an initial pattern of mature heterocysts [9]. PatS and HetN do not require the other for activity, and in filaments lacking both PatS and HetN nearly all cells differentiate into heterocysts, suggesting that PatS and HetN are the two predominant inhibitors of differentiation [7]. When grown with combined nitrogen, all cells of filaments have a low level of HetN in thylakoid and cytoplasmic membranes, but upon removal of combined nitrogen to induce differentiation, HetN is degraded [17]. An increase in *hetN* mRNA is seen 12 h after the induction of differentiation [4], at about the time that *de novo* pattern formation is complete and cells commit to differentiation. After this time, HetN protein and the expression of *hetN* are found exclusively in developing heterocysts [9,17]. HetN protein is predicted to be a member of the short-chain alcohol dehydrogenase family [5], but within the sequence of HetN is the PatS-5 sequence, RGSGR. The mechanism of the inhibition of differentiation by HetN is still unclear but, as for that of PatS, appears to include blockage of

the activity of *hetR* [9,17]. Recent experimental evidence which has shown that HetN, like PatS, limits the levels of HetR in a concentration-dependent manner and affects the levels of HetR over different spatial ranges when expressed in source cells [23] highlights the need to include HetN in the model system of equations describing heterocyst patterning.

2. Model equations

We introduce a set of differential equations to model the process of pattern maintenance over a large time scale where the interactions of HetR, PatS, and HetN are the main factors in the formation of new heterocysts between existing heterocysts as the spatial domain enlarges by cell division. Unlike Meinhardt's conceptual outline of pattern maintenance with a single inhibitor [19], the model includes two inhibitors. Figure 2 is a schematic of molecular interactions incorporated in the model that have been shown experimentally to regulate differentiation of heterocysts.

The maintenance stage occurs when several first-generation heterocysts have formed during the initial spatial patterning stage. During this maintenance stage, the following underlying assumptions are made:

- (a) HetR represents the activator, and HetN and PatS represent the inhibitors.
- (b) Initially, existing heterocysts produce HetN while PatS is present at a baseline level.
- (c) HetR and PatS increase at their respective basal expression rates.
- (d) HetR, PatS and HetN decrease at their respective basal degradation rates.
- (e) PatS and HetN diffuse freely from cell to cell.
- (f) HetR does not diffuse from cell to cell.
- (g) The growth of HetR occurs when the level of HetN is low.
- (h) HetR promotes the growth of itself and PatS.
- (i) PatS inhibits the growth of HetR.
- (j) Growth of HetN is triggered at a threshold level of HetR.
- (k) Heterocysts do not divide. Vegetative cells do divide.

The model equations consist of three conservation equations that describe the local change of the concentration of the activator HetR $R(x, t)$ and the two diffusive inhibitors PatS $S(x, t)$ and HetN $N(x, t)$, respectively. The quantities t and x are the respective temporal and spatial variables. Descriptions of all parameters are summarized in Table 1.

Conservation equation for R:

$$\frac{dR}{dt} = \alpha_R - \kappa_R R + \frac{\beta_R R^n}{(K_S + S)(K_R^n + R^n)} + \mu(R_e - R)^2(N_c - N - \eta S). \quad (1)$$

The local change of R per unit time is equal to the net change per unit time from constant basal expression α_R , natural degradation $\kappa_R R$, saturated positive feedback $\beta_R R^n / (K_S + S)(K_R^n + R^n)$ with Hill coefficient $n = 2$, and the inhibition by PatS and HetN. More specifically, the quantity

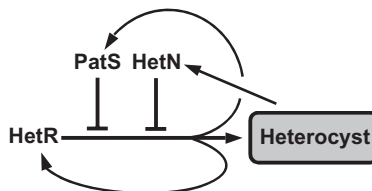


Figure 2. Schematic of the molecular interactions regulating heterocyst differentiation and patterning.

Table 1. Description of parameters.

| Parameter | Description |
|------------|---|
| α_R | Basal expression rate of HetR |
| κ_R | Natural degradation rate of HetR |
| β_R | Saturation rate of HetR positive feedback |
| n | Hill coefficient of HetR positive feedback |
| K_R | HetR concentration for half-maximal HetR activation |
| K_S | PatS coefficient for the inhibition of transcriptional activation by HetR |
| μ | Proportionality constant for activation/inhibition switch |
| η | Relative scalar for inhibitor PatS |
| α_S | Basal expression rate of PatS |
| κ_S | Natural degradation rate of PatS |
| β_S | Saturation rate of PatS positive feedback |
| D_S | Diffusion coefficient of PatS |
| κ_N | Natural degradation rate of HetN |
| β_N | Saturation rate of HetN positive feedback |
| D_N | Diffusion coefficient of HetN |
| ρ | Proportionality constant for the growth of the filament |

$K_S + S$ in the positive feedback term accounts for inhibition by PatS of transcriptional activation by HetR hence the higher the value of S , the smaller the positive feedback term. The Hill coefficient is set at 2 to reflect dimer formation by HetR. The term $\mu(R_e - R)^2(N_c - N - \eta S)$ models the impact of the two inhibitors HetN and PatS on HetR. When the combined inhibitor value is greater than some critical value N_c , $\mu(R_e - R)^2(N_c - N - \eta S) < 0$, HetN and PatS inhibit the growth of HetR. When the combined inhibitor value is smaller than N_c , the HetR concentration increases. The closer R is to R_e , the smaller the rate of this increase. No experimental evidence exists for the diffusion of HetR, and thus we assume the activator does not diffuse in our model.

Conservation equation for S :

$$\frac{\partial S}{\partial t} = \alpha_S - \kappa_S S + \frac{\beta_S R^n}{(K_S + S)(K_R^n + R^n)} + D_S \frac{\partial^2 S}{\partial x^2}. \quad (2)$$

The local change of S per unit time is equal to the net change per unit time from constant basal expression α_S , natural degradation $\kappa_S S$, activation by HetR in a saturated form similar to that in Equation (1) but with a different proportionality coefficient β_S , and diffusion of PatS with diffusion coefficient D_S .

Conservation equation for N :

$$\frac{\partial N}{\partial t} = -\kappa_N N + \frac{\beta_N R^n}{(K_S + S)(K_R^n + R^n)} + D_N \frac{\partial^2 N}{\partial x^2}. \quad (3)$$

The local change of N per unit time is equal to the net change per unit time from natural degradation $\kappa_N N$, activation by HetR in a saturated form with proportionality constant β_N , and diffusion of HetN with diffusion coefficient D_N . There is no basal expression of HetN to reflect the absence of HetN in vegetative cells after removal of combined nitrogen.

The growth equation for filament:

$$\frac{dL}{dt} = \rho L. \quad (4)$$

As the non-heterocyst cells divide, the field length grows. We assume that the field length L grows at a rate that is proportional to L . This assumption is consistent with recent models of cell growth [10]. This proportionality constant is denoted as ρ .

We use zero-flux (reflective) boundary conditions for PatS and fixed boundary conditions for HetR and HetN. In this case, we assume that heterocysts are located at the end points and the concentrations of HetR and HetN remain as constants at the end points, reflecting the observation that HetR continues to be transcribed at an induced rate in mature heterocysts [6].

3. Non-dimensionalization of the model

We non-dimensionalize the model equations to reduce the number of parameters and to facilitate the numerical solution. For simplicity, we retain the notations for the model variables and independent variables. We non-dimensionalize the model variables: $R \rightarrow R/K_R$, $S \rightarrow S/K_S$, $N \rightarrow N/[N]$, $t \rightarrow t/[T]$, and $x \rightarrow x/[L]$, where $[N] = 10N(0, 0)$. We choose the time scale $[T]$ to correspond to the time length (12 h) when a filament doubles its initial length. $[L]$ is set as the length of a filament with 10 cells (30–40 μm) such that the initial length of the non-dimensionalized filament is 1, that is, the initial spatial domain is $[0, 1]$. We select a Hill coefficient for $n = 2$ to reflect the experimental observation of dimer formation by HetR.

As the filament grows, the non-dimensional initial domain expands from $[0, 1]$ to $[0, L]$, where L increases with time. Due to the difficulty in mathematical analysis and numerical simulation on a changing spatial domain, we map the model equations with the changing domain size $[0, L]$ to a set of equations with fixed domain size $[0, 1]$ as in previous studies [3,10]. For an analysis of the results, they are mapped back to the domain of $[0, L]$. Hence, it follows that Equations (5)–(8) are non-dimensionalized model equations. L^2 in Equations (5)–(7), $-\rho R$ in Equation (5), $-\rho S$ in Equation (6), and $-\rho N$ in Equation (7) are introduced by mapping the changing domain to the fixed domain $[0, 1]$. All parameters in Equations (5)–(8) represent the respective non-dimensional coefficients. The detailed definition of these parameters is provided in Table 2.

$$\frac{dR}{dt} = a_R - c_R R + \frac{b_R R^2}{(1+S)(1+R^2)} + u(R_e - R)^2(N_c - N - vS) - \rho R, \quad (5)$$

$$\frac{\partial S}{\partial t} = a_S - c_S S + \frac{b_S R^2}{(1+S)(1+R^2)} + \frac{d_S}{L^2} \frac{\partial^2 S}{\partial x^2} - \rho S, \quad (6)$$

$$\frac{\partial N}{\partial t} = -c_N N + \frac{b_N R^2}{(1+S)(1+R^2)} + \frac{d_N}{L^2} \frac{\partial^2 N}{\partial x^2} - \rho N, \quad (7)$$

$$\frac{dL}{dt} = \rho L. \quad (8)$$

Table 2. Definition of non-dimensional parameters.

| | | |
|---------------------------------|------------------------------------|------------------------------------|
| $a_R = \frac{\alpha_R[T]}{K_R}$ | $c_R = \kappa_R[T]$ | $b_R = \frac{\beta_R[T]}{K_S K_R}$ |
| $u = \mu K_R[N][T]$ | $R_e \rightarrow \frac{R_e}{K_R}$ | $N_c \rightarrow \frac{N_c}{[N]}$ |
| $v = \frac{\eta K_S}{[N]}$ | $\rho \rightarrow \rho[T]$ | $a_S = \frac{\alpha_S[T]}{K_S}$ |
| $c_S = \kappa_S[T]$ | $b_S = \frac{\beta_S[T]}{K_S^2}$ | $d_S = \frac{D_S[T]}{[L]^2}$ |
| $c_N = \kappa_N[T]$ | $b_N = \frac{\beta_N[T]}{K_S K_N}$ | $d_N = \frac{D_N[T]}{[L]^2}$ |

4. Model results

A finite difference successive over-relaxation (SOR) method is used to solve the model equations numerically on a uniform grid. The numerical solutions simulate the pattern formation with the associated formation of a new heterocyst. With increasing time, the cells between the two heterocysts divide, and the spatial domain increases. The level of the concentration of HetN in the mid-section decreases. We seek to investigate pattern maintenance and select the initial conditions assuming some preliminary heterocyst formation. In particular, the patterning follows from the initial configuration of two heterocysts formed at the ends of the spatial domain.

We note that the experimental values for most of the biological parameters are unavailable and previous studies [10,15] have used values related to regimes of mathematical interest. However, estimates for a range of values of the diffusion coefficient for an inhibitor were made in [11] based on experimental observations and scaling arguments. These provided our initial estimate of diffusion coefficient of PatS and the upper limit of the potential diffusion coefficient value for HetN. In addition, we have used the standard linear diffusion-driven instability analysis [21] to determine the pattern formation conditions and parameters. This includes examining the growth and decay of each model variables, finding steady states of Equations (5) and (6), linearizing these equations, and determining the conditions for the diffusion-driven growth of HetR. Numerical experiments were performed using these conditions as well as estimates of the relative order magnitude of the other coefficients and parameters through a range of diffusion coefficient values to find robust regions of pattern formation. For pattern maintenance, the relative difference of the diffusion of the two inhibitors is of particular interest and we believe that ongoing experiment efforts will provide more specific values for diffusion parameters.

After the *de novo* formation of heterocysts, the concentration of HetR is at its positive equilibrium at these heterocyst locations and at its baseline level elsewhere. At this stage, heterocysts do not promote the growth of PatS, and PatS is at its baseline level, consistent with the observation that the expression of *patS* after differentiation of an initial pattern of cells is similar to the baseline level seen in cells prior to the induction of differentiation [29]. The concentration of HetN is high at the heterocyst locations and it diffuses to the neighbouring cells to inhibit the growth of HetR. Moreover, for the maintenance case, we assume the initial distribution of HetN follows a diffusion-mediated ‘bowl-shaped’ curve with higher values near the locations of heterocysts: $N(x, t) \propto (1/2)^{-4(x-0.5)^2}$. The graphs of the initial distributions are shown in Figure 3(a), where R is the solid curve, N is the dotted curve, and S is the dashed curve. The non-dimensionalized parameter values are $a_R = 0.001$, $c_R = 0.4$, $b_R = 2.0$, $u = 2 \times 10^4$, $R_e = 0.13$, $N_e = 0.0125$, $v = 0.2$, $\rho = 0.1$, $a_S = 0.01$, $c_S = 1.0$, $b_S = 20.0$, $d_S = 1.5$, $c_N = 1.8$, $b_N = 1.0$, and $d_N = 0.15$. As N decreases below a critical value, the concentration of HetR increases in this mid-section and such an increase triggers the growth of PatS. Subsequently, PatS diffuses to the surrounding neighbourhood to inhibit a wide range growth of HetR such that only one heterocyst is generated in the mid-section. The distributions at length $L = 1.8$ at $t = 10.95$, $L = 1.9$ at $t = 11.49$, and $L = 2$ at $t = 12$ are shown in Figure 3(a)–(d), respectively. The overall shape of R does not change from $L = 1.95$ to 2. The formation of the new heterocyst begins when the length of the filament is close to doubling its initial length with one heterocyst generated. Figure 3(e) is the contour map of R at $L = 2$ and $t = 12$ that shows the gradient of R . Although one heterocyst will be generated at the centre, the neighbouring cells around it show small amounts of HetR concentration.

The difference of the two inhibitors’ diffusive rates plays an important role. HetN must diffuse slower than the growth rate of the length of the filament so that the concentration of HetN in the middle drops below the critical value to induce the growth of HetR as the length between the two heterocysts grows to about a 20-cell length. If we choose larger diffusive rates for HetN ($d_N = 0.5$), the HetN concentration does not drop below the critical value and no heterocyst is

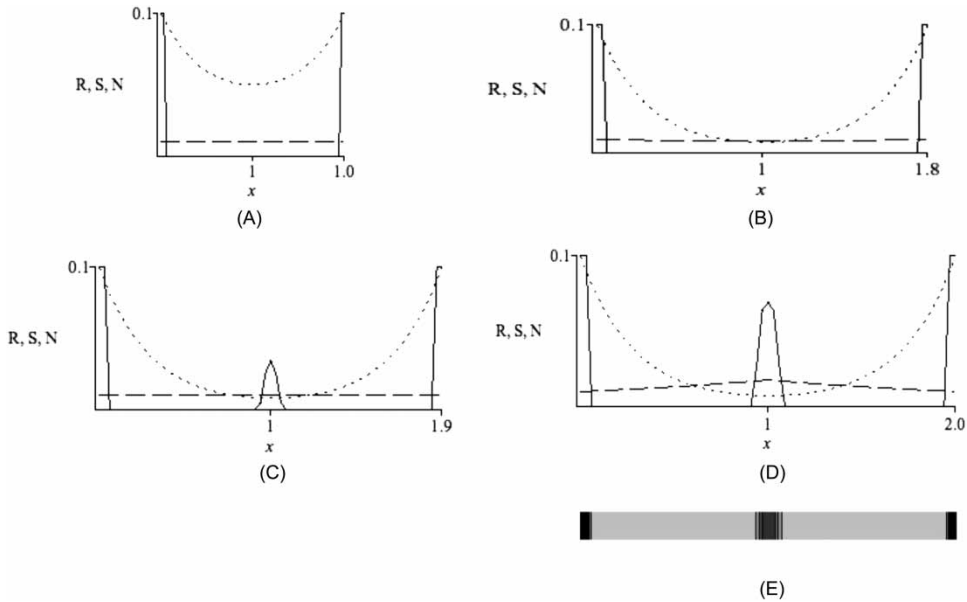


Figure 3. Distribution curves for the one non-diffusive activator and two-diffusive inhibitor model (5)–(8). The horizontal axis represents the non-dimensionalized spatial variable x . The vertical axis represents the non-dimensionalized model variables R, S , and N . The spatial distributions of R, S , and N are represented by solid, dashed, and dotted curves, respectively. (a) The initial distributions of R, S , and N at the length of $L = 1$ and time $t = 0$; (b) the distributions at $L = 1.8$ and $t = 11.60$; (c) the distributions at $L = 1.9$ and $t = 11.77$; and (d) the distributions at $L = 2$ and $t = 12$. As non-heterocyst cells divide, the field length (L) grows. The inhibitor $HetN$ diffuses slowly, and its concentration level decreases in the mid-section of the field. As a result, $HetR$ increases in the mid-section right before $L = 1.8$, and the growth of $HetR$ triggers the growth of the second inhibitor, $PatS$. $PatS$ diffuses faster than $HetN$ to maintain the growth of $HetR$ spatially for inducing the differentiation of one heterocyst. (e) Contour map of $HetR$ concentration in (d).

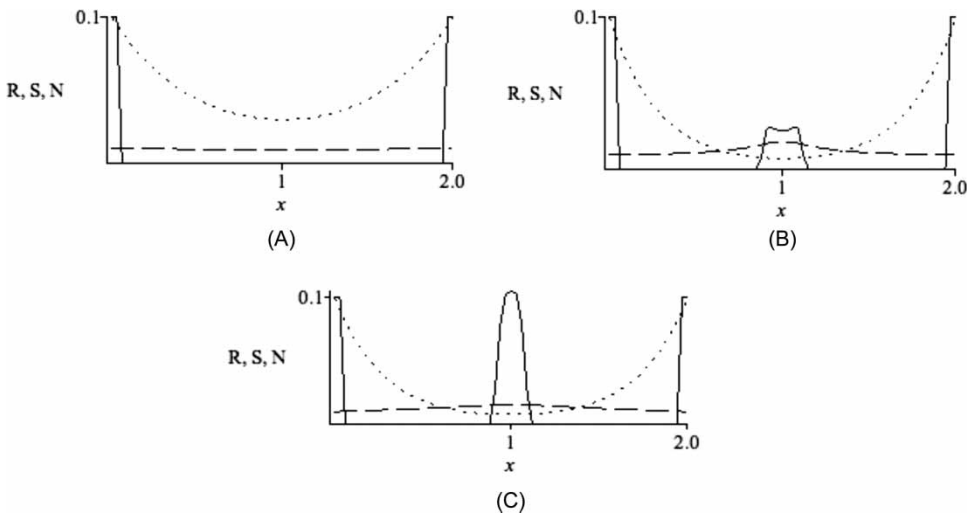


Figure 4. Distribution curves for the one non-diffusive activator and two-diffusive inhibitor model (5)–(8) at $L = 2$. (a) Large diffusive rate of $HetN$, $d_N = 0.5$, the concentration of $HetN$ in the mid-section remains relatively high. No new heterocysts are generated. (b) $PatS$ and $HetN$ both have the same low diffusive rate, $d_N = d_S = 0.15$. The magnitude of $HetR$ concentration is relatively low but occurs in multiple cells. (c) When the impact of $PatS$ on $HetR$ is reduced from $v = 0.2$ in Figure 3 to $v = 0.15$, the growth of $HetR$ in the mid-section (c) is twice as wide as that in Figure 3(d). Two heterocysts can be induced.



Figure 5. Heterocyst location mapping. (a) One heterocyst is initially located at each end of the filament ($L = 1$ and $t = 0$, corresponding to Figure 3(a)). (b) One heterocyst is generated in the mid-section as the length of the filament doubles ($L = 2$, $t = 12$ and $v = 0.2$, corresponding to Figure 3(d)). (c) Two heterocysts are generated in the mid-section when the impact of PatS on HetR is reduced ($L = 2$, $t = 12$ and $v = 0.15$, corresponding to Figure 4(c)).

generated (Figure 4(a)). On the other hand, the diffusive rate of PatS should be larger than that of HetN. Figure 4(b) shows the simulation result where $d_N = d_S = 0.15$. The concentration of PatS in the middle of the filament is relatively high, that is, the small diffusive rate of PatS causes a small elevated HetR concentration appearing in multiple cells.

With the model, we can also investigate the effect of each term on pattern formation. For example, as we reduce the inhibitor effect of PatS on HetR by using smaller value of η ($v = 0.15$), the profile of the HetR concentration curve in the mid-section becomes wider (Figure 4(c), solid curve). This simulates the biological situation observed when more than one heterocyst is generated to give a Mch phenotype when *patS* is mutated (Figure 1(c)).

Assuming the threshold value of HetR in triggering differentiation into a heterocyst is 50% of its equilibrium value of HetR in a heterocyst, the spatial patterns corresponding to Figure 3(d) and Figure 4(c) are given in Figure 5(b) and (c), respectively, where one new heterocyst is formed in Figure 5(b) and two heterocysts are formed in Figure 5(c) due to the reduction (mutation) of PatS. The corresponding initial condition is shown in Figure 5(a). Black areas represent the locations of the heterocysts and grey areas correspond to the vegetative cells.

5. Discussion

Most reaction–diffusion-based models for biological pattern formation have incorporated a diffusive activator together with a diffusive inhibitor. When the inhibitor diffuses faster than the activator, non-homogeneous spatial patterns can be generated by introducing an initial random disturbance about the homogeneous steady state of the system. Such models have been successful in generating spatial patterns that mimic those in nature. There is, however, no evidence for such diffusion between cells for HetR in *Anabaena*. In contrast, experimental introduction of extra copies of the gene encoding HetR or overactive forms of HetR to only some cells of filaments to create genetic mosaics does not increase the likelihood of differentiation of adjacent cells. Similarly, genetic mosaic filaments in which only some cells of filaments contain the gene for HetR translationally fused to that for the green fluorescent protein (GFP) indicate that HetR–GFP is present only in cells that contain the genetic construct. Both experiments suggest that HetR does not diffuse from cell to cell.

The new model presented here incorporates two inhibitors. Growing experimental evidence indicates that PatS diffuses from cell to cell to regulate pattern formation [28]. Although, the evidence and understanding of the diffusion of HetN is less extensive, all the available experimental evidence points in this direction. Recent experimental evidence indicates that both PatS and HetN affect levels of HetR in cells 10–20 away from cells producing PatS or HetN, either as heterocysts

or cells manipulated to overexpress one of the inhibitors [23]. However, unlike for PatS, it is unknown whether it is the HetN protein or a product of its putative catalytic activity that is responsible for its inhibitory activity. Mutation of the PatS-5 sequence in HetN was reported to have no effect on the activity of HetN [17]. In addition to R132K and R136L substitutions, the report claims that G134S and S135D substitutions were made and had no effect. This is puzzling because the HetN sequence is S134 and G135, not G134 and S135 as indicated, so what substitutions were actually made is unclear. Regardless, the model is relevant and applicable if HetN itself or a HetN-dependent signal acts as a diffusible inhibitor, or if HetN senses an as-yet undiscovered inhibitory signal. It is our hope that further experiments in conjunction with further comparisons to refined models building upon this work will help address these outstanding issues in the field.

Pattern maintenance is accurately reproduced with numerical solutions of the model. At the outset of the maintenance stage of patterning, the heterocysts at the ends of the field are the main source of the inhibitor HetN. The diffusion of HetN results in a bowl-shaped concentration distribution. Together with PatS, HetN inhibits the growth of HetR. As cells divide, the field grows such that, combined with the small diffusive rate of HetN, the concentration of HetN in the mid-section of the field decreases. This fosters a rise in the growth of HetR in the mid-section. The growth of HetR triggers the growth of PatS. As PatS diffuses to the neighbourhood and inhibits the growth of HetR in the surrounding neighbourhood, the growth of HetR is limited to the mid-section where only one new heterocyst is generated.

Our model with one non-diffusive activator and two diffusive inhibitors reproduces maintenance of the pattern of heterocysts observed in *Anabaena*. The unequal diffusion rates of the two inhibitors play a role in the maintenance patterning. The PatS concentration grows rapidly, corresponding to the growth of HetR. PatS has a faster diffusive rate than HetN ($d_S > d_N$) and diffuses from the mid-section towards the end points. This eliminates growth of HetR around the mid-section permitting one heterocyst to differentiate. The slower diffusive rate of HetN ($d_N < d_S$) regulates the spacing (approximate every 10-cell length) for initiating a new heterocyst.

The simplified model of the regulation of maintenance of heterocyst patterning presented here consists of only three variables: HetR, PatS, and HetN. Although the model does not account for levels of fixed nitrogen in cells, we believe it is consistent with the maintenance patterning process. The diffusion of the products of fixed nitrogen from heterocysts is an obvious potential factor affecting the placement of new heterocysts, but it appears that although fixed nitrogen levels are involved in the induction of differentiation by a filament, they are not intimately involved in pattern maintenance. Mutants that lack nitrogenase activity still have a normal pattern of heterocysts, and when an alternate nitrogenase is active in all vegetative cells and supplies sufficient fixed nitrogen for robust growth in *Anabaena variabilis*, initial pattern formation and maintenance are normal [24].

6. Conclusion

The one-dimensional pattern of only two cell types made by *Anabaena* is, arguably, one of the simplest developmental patterns, making it an ideal system for the elucidation of the minimal developmental genetic requirements for the formation of a sustainable pattern of terminally differentiated cell types in an organism. Despite recent advances in uncovering the molecular interactions that regulate patterning, complementary mathematical modelling efforts have been both sparse and limited. We have developed a model, based on updated knowledge of patterning proteins, consisting of two partial differential equations and two ordinary differential equations. The equations describe two inhibitors, PatS and HetN, which undergo diffusion; an activator,

HetR, which does not; and the growth of the spatial domain. The set of equations is solved numerically for a growing cell domain to investigate the case of pattern maintenance. Under these conditions, the model predicts the formation of a single, new heterocyst. Analytical analysis of the equations including a stability analysis should yield further insights, and indeed, this is a topic for future work. We believe that continued developments in the mathematical modelling of this system in conjunction with complementary experimental efforts should enhance our understanding of periodic patterning in *Anabaena* and related systems.

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